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Identification and membrane localization of electrogenic sodium bicarbonate cotransporters in Calu-3 cells

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Abstract

Cystic fibrosis (CF) is a severely life-shortening genetic disease resulting from mutations in the gene for the cystic fibrosis transmembrane conductance regulator (CFTR). Impaired bicarbonate secretion is a key component of CF-related pancreatic disease, but the role of impaired bicarbonate secretion in CF lung disease is less well understood. The submucosal glands of the conducting airways produce and secrete a complex airway surface liquid that lines the airway epithelium and plays a significant role in mucociliary clearance. The serous cell is the predominant cell type of the submucosal gland and a predominant site of CFTR expression. Calu-3 cells are a model of airway submucosal gland serous cells that demonstrates vectorial bicarbonate secretion in response to elevations in cAMP. Based on previously published measurements of unidirectional ion flux, pharmacological inhibition of short-circuit current and ion substitution studies, one can hypothesize the existence of an electrogenic sodium bicarbonate cotransporter (NBC) in the basolateral membrane of Calu-3 cells that mediates bicarbonate entry from the interstitium. To test this hypothesis, we performed reverse-transcriptase PCR, western blotting, and surface biotinylation to identify and localize electrogenic NBCs in Calu-3 cells. Our data demonstrate that both pNBC1 and NBC4 mRNAs can be identified and that their protein products are expressed at the basolateral membrane of polarized Calu-3 cells. These data suggest that these transporters contribute to regulated bicarbonate secretion across Calu-3 cells and perhaps human airway submucosal glands.

Keywords: Sodium bicarbonate cotransporter; Bicarbonate; Cystic fibrosis; Calu-3 cell

1. Introduction

Cystic fibrosis (CF) is a severely life-shortening genetic disease resulting from mutations in the gene for the cystic fibrosis transmembrane conductance regulator (CFTR). Patients with CF may suffer from dysfunction of almost every organ whose function depends on regulated anion secretion, most prominently the pancreas and lungs. In the pancreas of patients with CF, impaired alkalinization of pancreatic secretions leads to premature activation of pancreatic enzymes eventuating in autolysis, inflammation, fibrosis, and pancreatic exocrine dysfunction in greater than 90% of CF patients. In the lungs of patients with CF, decreased CFTR-mediated chloride (Cl^-) conductance and increased sodium (Na^+) conductance at the apical membrane of airway epithelial cells lowers the height of the periciliary fluid layer (PCL) resulting in apposition of airway mucus to the epithelium and subsequent mucus stasis [1]. The resulting chronic infection and inflammation are the major cause of morbidity and mortality in CF. However, abnormal Cl^- conductance may not be the only physiological deficit attributable to decreased CFTR function.

In addition to its Cl^- permeability, CFTR is permeable to other anions, the physiologically important permeation being to bicarbonate (HCO_3^-) [2–5]. As noted above, pancreatic dysfunction secondary to reduced HCO_3^- secretion is a hallmark of CF pathophysiology in the pancreas. The role of altered HCO_3^- secretion in the pathogenesis of CF lung disease is less clear; however, impaired HCO_3^- secretion has been implicated in the pathogenesis of CF lung disease [6]. For example, evidence suggests that changes in pH have a direct

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effect on the viscosity of respiratory mucus and ciliary beat frequency [7,8]. Even more compelling, two recent papers have shown that submucosal glands from patients with CF secrete a more acidic fluid than those from non-CF controls [9], and that CF epithelia do not alkalinize ASL appropriately in response to elevated intracellular cAMP [10]. These findings suggest that the ASL of CF patients is altered in both its production by submucosal glands and its modulation by airway epithelia. Therefore, understanding transepithelial HCO_3^- secretion is likely of critical importance for our understanding CF lung pathophysiology.

Submucosal glands are responsible for the majority of fluid and mucus secretion in human airways. The submucosal glands are comprised approximately 60% by volume of serous cells, and they are the predominant site of CFTR expression in the lung [11,12]. Selected from a lung adenocarcinoma, Calu-3 cells are a human airway serous cell line identified by Wine, Widdicombe, and coworkers that have many of the biological and electrophysiological properties of human airway serous cells [13]. They are a good model for the study of CFTR because they form confluent monolayers that express high levels of endogenous CFTR, and they respond to cAMP- and Ca^{2+}-mediated agonists with changes in net ion movement [13]. In particular, in response to forskolin, an activator of adenylyl cyclase, Calu-3 cells have been shown to secrete HCO_3^- by a Cl^- independent, serosal Na^+-dependent, and serosal stilbene-sensitive, electrogenic mechanism [14]. Together, these observations suggest the hypothesis that electrogenic sodium bicarbonate cotransporters (NBCs) will be found in the basolateral membrane of Calu-3 cells. Therefore, we performed reverse-transcriptase PCR (RT-PCR), western blotting, immunoprecipitation, and membrane-specific biotinylation to demonstrate that Calu-3 cells express electrogenic NBCs and that these NBCs are specifically located in the basolateral membrane.

2. Methods

2.1. Cell culture

Calu-3 cells were purchased from ATCC and grown as previously described [14]. Briefly, cells were grown to confluence in 25 cm^2 flasks in DMEM/F-12 supplemented with 15% FBS and 1% penicillin/streptomycin. Cells were removed with trypsin and seeded onto 1.1 cm^2 polycarbonate inserts (Snapwell inserts, Costar, Corning, NY). On day 1 apical medium was removed creating an apical air–liquid interface. Cells were subsequently fed only on the basolateral side. Basolateral media was refreshed every 48 to 72 h and the day prior to any experiment. Filters were used only after the apical surface remained free of media, usually between 14 and 28 days after establishment of an apical air interface.

2.2. Reverse-transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from mature, polarized Calu-3 cells by TriZol (Invitrogen, Carlsbad, CA) extraction according to manufacturer's instructions. First-strand synthesis was performed using oligo-dT primers and Superscript III reverse transcriptase (Invitrogen). Unique primer pairs were generated for representatives of each of four NBC genes (Table 1): pNBC1 (AF011390, SLC4A4), NBC2 (AF047033, SLC4A7), NBC3 (AF107099, SLC4A8), and NBC4c (AF293337, SLC4A5). PCR was carried out with Platinum Taq Supermix (Invitrogen) using a three-step protocol with a melting temperature of 94 °C, an annealing temperature of 55 °C, and an extension temperature of 68 °C for 30 cycles. PCR products were separated on a 1% agarose gel and visualized with ethidium bromide staining. PCR reactions generating a single PCR product were subcloned into PCR4-TOPO (Invitrogen) and sequenced for verification. Full-length PCR products from pNBC1 mRNA was also obtained using oligo-dT primers for first-strand synthesis and an extension time of 3 min during the PCR reaction.

2.2.1. Generation of polyclonal antibodies

The sequence CKSYRRRRRHKRKTGHKEKKEKE was used to generate the polyclonal antibody against pNBC1. The peptide was coupled to Imject® maleimide activated mcKLH according to manufacturer's directions (Pierce, Rockford, IL). On day one, an emulsion was made with 500 μg coupled peptide in complete Freund's adjuvant and equally distributed through five subcutaneous sites. On the fourteenth and twenty-first day, coupled peptide was emulsified in incomplete Freund's and injected as before. Blood was collected from non-immunized rabbits and 2 weeks after the final immunization. Blood was collected via a 21-gauge butterfly needle inserted into the medial ear artery. Blood was allowed to clot at room temperature, and was then centrifuged at 10,000×*g* for 10 min. Serum was harvested and stored at −80 °C. The sequence RRRFPDQKEC was used to generate the antibody against NBC4. The NBC4 antibody was commercially generated and antisera supplied by Affinity Bioreagents (Golden, CO).

2.2.2. Western blotting

Calu-3 filters were lysed in buffer containing 1% Triton 20, 10% Glycerol, and 25 mM HEPES with protease inhibitors (Complete protease inhibitors, Roche). Samples were normalized for total protein by BCA assay (Pierce, Indianapolis, IN), loaded on to 12% polyacrylamide gels, and resolved by electrophoresis. Proteins were transferred on to nitrocellulose membranes and non-specific binding was blocked with 5% nonfat milk in tris-buffered saline with 1% Tween 20. Membranes were probed with antibody to pNBC1 and NBC4. Bands were visualized by autoradiography after conjugation with an HRP-labeled, goat anti-rabbit secondary antibody (Amersham, Piscataway, NJ).

2.2.3. Immunoprecipitation

Calu-3 filters were rinsed twice with phosphate buffered saline, then scraped into hypotonic lysis buffer (in mM: 50 Tris–HCl, 120 NaCl, 2 Empigen BB, and 0.5% NP-40) and rotated at 4 °C for 2 h. Lysates were centrifuged at 13,000×*g* for 3 min and supernatant was recovered. Anti-pNBC1 or anti-NBC4 antibody (200 μg) was added, and supernatants were rotated overnight at 4 °C. Protein A beads were washed in hypotonic lysis buffer and added to the antibody/antigen complex, and rotated overnight at 4 °C. The bead complex was centrifuged, washed three times in hypotonic lysis buffer and once in Tris-buffered saline. Finally, Laemmli buffer was added, samples were boiled for 3 min, resolved by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) (prewetted in methanol) in Towbin's transfer buffer (25 mM Tris, 192 mM glycine, and 20%

Table 1
Reverse-transcriptase PCR amplicons and primers

Isoform	GenBank ID	Amplicon	Forward primer	Reverse primer
pNBC1	AF011390	186–934	AGA AGG CCA CCA TAC CAT TTA CAT CG	ACT TAT TCT TCA GCT GGT CCT TCT CCG
pNBC1 (full length)	AF011390	118–3357	ATG GAG GAT GAA GCT GTC	TCA GCA TGA TGT GTG GC
NBC2	AF047033	175–1134	GAG GAT GTT GAA GAT GGC GGT G	ACC AAA AAG CCG TCC AGT CCT C
NBC3	AF107099	297–1195	ACC ATC TCA GCG TGT TCA GTT CAT	TCA CCT GGT CTA GGA ACT CAT CAA TC
NBC4	AF293337	3210–3743	GTG CTG TAC GGA GTC TTC CTC TA	CGG TCA AGT TCT GTG TCA CTG A

methanol, pH 8.3). Membranes were probed with antibody to pNBC1 and NBC4. Bands were visualized by autoradiography after conjugation with an HRP-labeled, goat anti-rabbit secondary antibody (Amersham).

2.2.4. Biotinylation of individual membranes

All reagents and cells were brought to 4 °C prior to the beginning of the experiment. Biotinylation of specific membranes was carried out according to the methods of Gottardi et al. [15]. Briefly, Calu-3 cells grown on Snapwell inserts were washed sequentially with serum-free media and then PBS with Ca^{2+} and Mg^{2+}. Following washes, either the apical or basolateral membrane was incubated twice consecutively for 25 min with biotin (1.5 mg/ml in 10 mM triethanolamine, 2 mM CaCl, 150 mM sucrose, pH 9). Cells were lysed in 150 mM NaCl containing 1% Triton X-100, 5 mM EDTA, 50 mM Tris (pH 7.5) and then centrifuged at 13,000×*g* for 10 min. Supernatants were transferred to a new microcentrifuge tube and 75 μL neutravidin beads (Pierce) were added. Supernatants with avidin were incubated overnight at 4 °C on an end-over-end rotator. Beads were collected by centrifugation and washed 3 times with lysis buffer, 2 times with 500 mM NaCl containing 5 mM EDTA and 50 mM Tris (pH 7.5), and once with 10 mM Tris (pH 7.5). Biotinylated proteins were eluted in 75 μL of Laemmli buffer and separated by SDS-PAGE as described for western blotting. Immunoblotting was performed with α-pNBC1, α-NBC4, or α-CFTR (polyclonal antibody 3195, generously provided by Professor Christopher R. Marino, M.D., University of Tennessee Health Science Center).

3. Results

3.1. RT-PCR

Using polarized Calu-3 cells for isolation of total RNA and oligo-dT for first-strand synthesis of cDNA, we were able to detect qualitatively mRNA from a representative member of each of four known NBC genes (Fig. 1). Each of the bands obtained correlated in size with the expected product. Furthermore, each band was subcloned into pCR4-Topo (Invitrogen) and verified by sequencing using M13 forward and reverse primers. The NBC1 product was homologous to the pancreatic isoform of NBC1 first cloned by Abuladze and colleagues [16]. The NBC2 product was homologous to the electroneutral, stilbene-insensitive NBC first cloned by Pushkin and colleagues (mNBC3) [17]. The NBC3 product was homologous to that cloned by Amlal and colleagues from brain (NBC-3). The NBC4 product was homologous to the electrogenic, stilbene-sensitive NBC first cloned by Sassani and colleagues (NBC4c) [18].

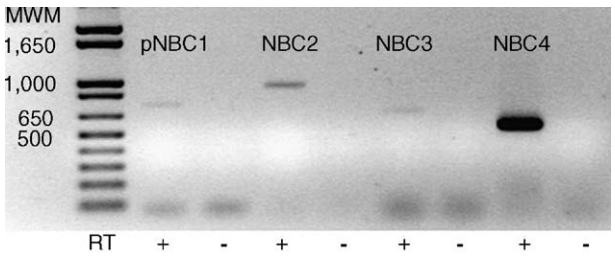

Fig. 1. RT-PCR demonstrates 4 NBC genes in Calu-3 cells. Total RNA was isolated from mature, polarized Calu-3 cells grown on permeable supports. First strand synthesis was carried out using oligo-dT primers and PCR was carried out with the primers shown in Table 1. Each band is shown next to a PCR reaction using untranscribed total RNA as the template. The identity of the individual bands was verified by sequence analysis. Note that gene products from each of four known NBC genes (SLC4A4, SLC4A5, SLC4A7, SLC4A8) can be detected.

Qualitatively, there was substantially more amplification of NBC4c than of cDNA from other NBC isoforms.

Because our prior work predicted the presence of an electrogenic NBC in the basolateral membrane, we designed primers to amplify in a single PCR reaction the coding regions of either pNBC1 or NBC4c, NBC genes whose protein products have been shown previously to be electrogenic in transport assays [16,18], and in the case of pNBC1, shown to be expressed in Calu-3 cells [19]. For these experiments we again used oligo-dT primers to preferentially amplify mRNA. Notably, we were able to amplify full-length cDNA for pNBC1 (Fig. 2), confirming the presence of this isoform despite the relatively faint band seen in the original RT-PCR. Sequencing of the NBC1 product verified homology to the published GenBank sequence for the coding region of pNBC1. We were unable to amplify full-length mRNA for NBC4c.[1]

3.2. Immunoblotting and immunoprecipitation

Our next set of experiments was designed to identify pNBC1 and NBC4 protein in Calu-3 cells. To do so we designed antibodies against peptide sequences from both pNBC1 and NBC4, using the respective amino-to carboxy-terminus sequences, CKSYRRRRRHKRKTGHKEKKEKE and RRR-FPDQKEC. The pNBC1 sequence is unique to the pancreatic isoform of the NBC1 gene, while the sequence used to generate the NBC4 antibody is shared between all of the known NBC4 isoforms, but not shared by other NBCs. In a first set of experiments, we performed immunoblotting on increasing amounts of protein from total cell lysates of mature, polarized Calu-3 cells. We demonstrated that our antibodies recognized target proteins of approximately 125 and 190 kDa for pNBC1 and NBC4, respectively (Fig. 3). To verify that the recognized proteins had undergone post-translational maturation in the Golgi apparatus, we performed deglycosylation experiments with endoglycosidase H (endo H) or peptide-N-glycosidase (PNGase). These experiments revealed that the recognized proteins were resistant to endo H, but sensitive to PNGase, consistent with post-translational N-glycosylation, i.e., consistent with a mature, membrane protein (Fig. 4a and b). Because the apparent molecular weight of pNBC1 in these experiments (~145 kDa) was slightly higher than that seen with immunoblotting alone, we performed immunoprecipitation in the presence of competing peptide antigen to demonstrate that the identified band represented pNBC1 (Fig. 4c).

3.3. Membrane specific biotinylation

Because the model of HCO_3^- secretion in Calu-3 cells proposed by Devor and colleagues [14] predicts the presence of an electrogenic NBC in the basolateral membrane but not the

[1] One possible explanation for the inability to obtain a full-length PCR product for NBC4 is the relatively high (60%) AT content of the 3' end, which imparts a low melting temperature for primers designed to anneal in that region. However, varying the melting temperature from 55 ° to as low as 50 °C did not result in successful amplification.

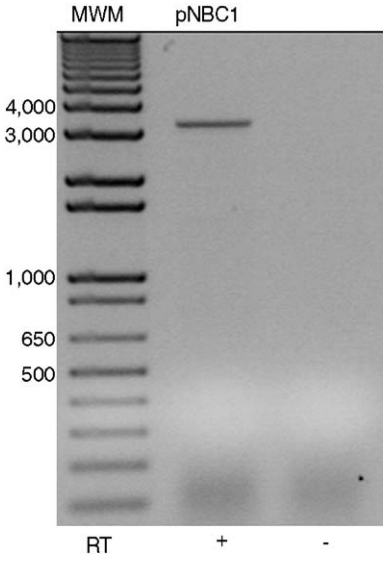

Fig. 2. Full-length pNBC1. Total RNA was isolated from mature, polarized Calu-3 cells grown on permeable supports. First strand synthesis was carried out using oligo-dT primers and PCR was carried out using a 5′-primer that started with the ATG start codon and a 3′-primer that ended with the TGA stop codon. Note that a single band was identified at the expected size of approximately 3.3 kB. Subcloning and sequence analysis verified the product as the full-length cDNA for pNBC1 (SLC4A4).

apical membrane, we were particularly interested in determining the membrane localization of the immunoblot detected NBCs. Therefore, we performed membrane-specific biotinylation using mature, polarized Calu-3 cells. We were able to detect both pNBC1 and NBC4 at the basolateral membrane of polarized Calu-3 cells. Furthermore, we were unable to detect either pNBC1 or NBC4 at the apical membrane of the same cells (Fig. 5). As surface biotinylation is strongly dependent on the pH, osmolarity and salt concentration of the biotinylation buffer [15], we wanted to ensure that our protocol was valid for biotinylation of apical membrane proteins. Therefore, we probed our biotinylated apical membrane samples for CFTR, which is abundantly expressed at the apical membrane of Calu-3 cells. We were able to detect robust CFTR expression at the apical membrane, consistent with pNBC1 and NBC4 being absent from the apical membrane of Calu-3 cells.

4. Discussion

There is agreement in the literature that Calu-3 cells transport HCO_3^- [14,19–21], but there is not a consensus regarding the mechanism of vectorial HCO_3^- transport [14,21]. The model of vectorial HCO_3^- secretion proposed by Devor and colleagues predicts the presence of an electrogenic NBC in the basolateral membrane, but not the apical membrane, of Calu-3 cells [14]. These data are supported by the findings that HCO_3^- secretion was prevented by removal of sodium from the serosal bathing solution and by the finding that HCO_3^- secretion was more sensitive to inhibition by DNDS in the serosal bath solution than to inhibition by acetazolamide [14]. The data presented here confirm the hypothesis that electrogenic NBCs reside at the basolateral membrane of Calu-3 cells and identify two candidate proteins, pNBC1 and NBC4.

First, the data demonstrate that mRNA from each of four currently known NBC genes is present in mature, polarized Calu-3 cells. Our data confirm the findings of Inglis and colleagues that pNBC1 is expressed in Calu-3 cells [19], but, in contrast, are also consistent with the presence of electroneutral NBCs in Calu-3 cells. This discrepancy may be the result of isolating RNA from mature, polarized Calu-3 cells, rather than Calu-3 cells grown on coverslips. Specifically with regards to electrogenic NBCs, we detected pNBC1 using primers targeted to the 5′ end of the coding region, and we detected NBC4c using primers targeted to the 3′ end of the coding region and the 3′ untranslated region (Table 1 and Fig. 1). NBC4 has been detected in human lung by Northern blot analysis [22], but in the original functional characterization of NBC4c, transcripts were not reported in the lung [18].

We were particularly interested in products from the NBC1 and NBC4 genes because the protein products from these genes are electrogenic [16,18,23]. Therefore, we attempted amplification of full-length mRNA for pNBC1 and NBC4c using primers that framed the respective open-reading frames (ORFs). We demonstrated that full-length pNBC1 mRNA can be amplified from RNA extracted from mature, polarized Calu-3 cells. Although we did not successfully amplify a full-length NBC4 isoform in a single RT-PCR reaction, we were able to amplify

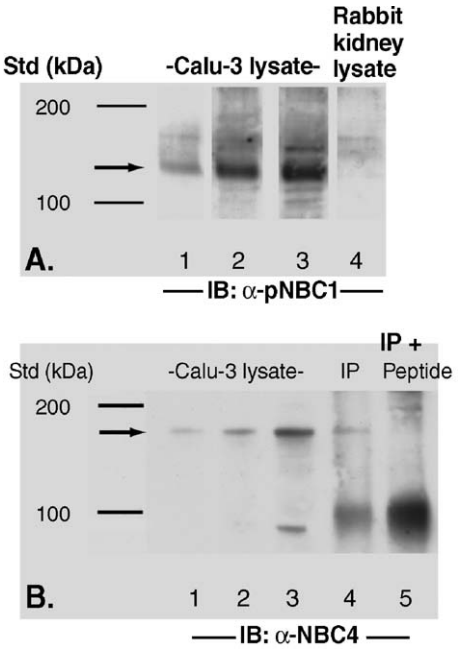

Fig. 3. Immunoblotting reveals pNBC1 and NBC4 in Calu-3 cells. Mature, polarized Calu-3 cells were lysed and total soluble protein in increasing amounts was loaded for SDS-PAGE followed by immunoblotting. (A) Immunoblotting with α-pNBC1 antibody; lane 1: 10 μg total protein; lane 2: 25 μg total protein; lane 3 50 μg total protein; lane 4: 50 μg total protein from rabbit kidney lysate. (B) Immunoblotting with α-NBC4 antibody: lane 1: 10 μg total protein; lane 2: 20 μg total protein; lane 3: 50 μg total protein; lane 4: total protein isolated following immunoprecipitation with α-NBC4 antibody; lane 5: total protein isolated following immunoprecipitation with α-NBC4 antibody in the presence of the peptide antigen.

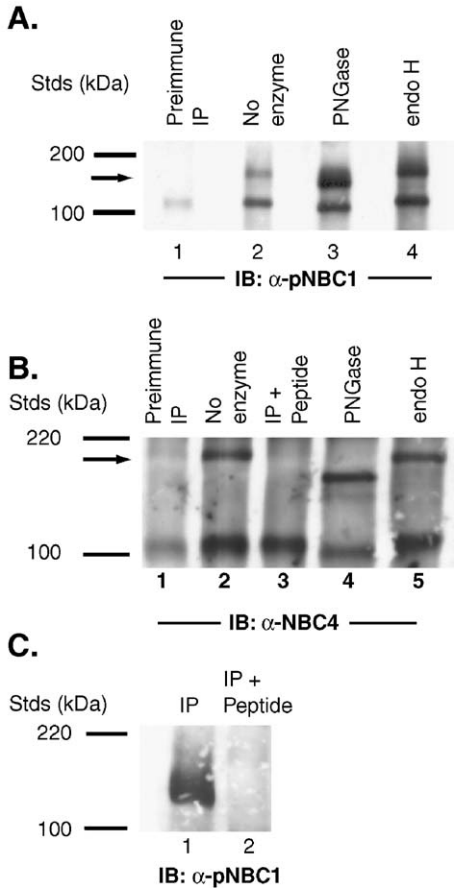

Fig. 4. Electrogenic NBCs in Calu-3 cells undergo post-translational modification. Calu-3 cell lysates were used for immunoprecipitation with either a-pNBC1 or a-NBC4. Immunoprecipitated proteins were processed with either endo H or PNGase followed by SDS-PAGE and immunoblotting. (A) Immunoprecipitation and immunoblotting with α-pNBC1 antibody: lane 1: immunoprecipitation using pre-immune serum; lane 2: no enzyme treatment; lane 3: PNGase treatment; lane 4: endo H treatment. (B) Immunoprecipitation and immunoblotting with α-NBC4 antibody: lane 1: immunoprecipitation using preimmune serum; lane 2: no enzyme treatment; lane 3: immunoprecipitation with α- NBC4 antibody in the presence of the peptide antigen; lane 4: PNGase treatment; lane 5: endo H treatment.

sequence homologous to the 3′ end and 3′-untranslated region of NBC4c using primers that are shared by NBC4a, NBC4b, NBC4c, and NBC4d, strongly suggesting that this is the NBC4 isoform expressed in Calu-3 cells.[1] However, we cannot completely eliminate the possibility that NBC4d (NM_133479) is expressed, because our 5′ primer had only 50% homology with the corresponding region of the NBC4d ORF. Our initial RT-PCR results suggested that NBC4 was more highly expressed in Calu-3 cells than pNBC1. However, the RT-PCR reaction was carried out using oligo-dT primers for first strand synthesis and the NBC4 primers were much closer to the 3′ end of their target transcript than were the pNBC1 primers, which were at the very 5′ end of their target transcript. Therefore, there was likely more cDNA available for amplication by the NBC4 primers than for the pNBC1 primers. This strategy was undertaken to allow the NBC1 primers to differentiate between the two NBC1 isoforms, which differ at the 5′ end of their respective transcripts [16], and to allow the NBC4 primers to differentiate between the various NBC4 isoforms, which differ primarily at the 3′ end of their respective transcripts [23].

To investigate if the identified transcripts underwent translation, we generated novel antibodies against pNBC1 and NBC4. Immunoblotting of whole Calu-3 cell lysates with affinity-purified antibodies confirmed the presence of protein products from pNBC1 and NBC4. The observed molecular weight of pNBC1 in these studies was approximately 125 kDa, in good agreement with the predicted molecular weight, 120 kDa, and previous studies of two proteins with high levels of homology to pNBC1, rb1NBC [24] and kNBC1 [25]. The observed molecular weight of NBC4 was approximately 190 kDa, greater than the

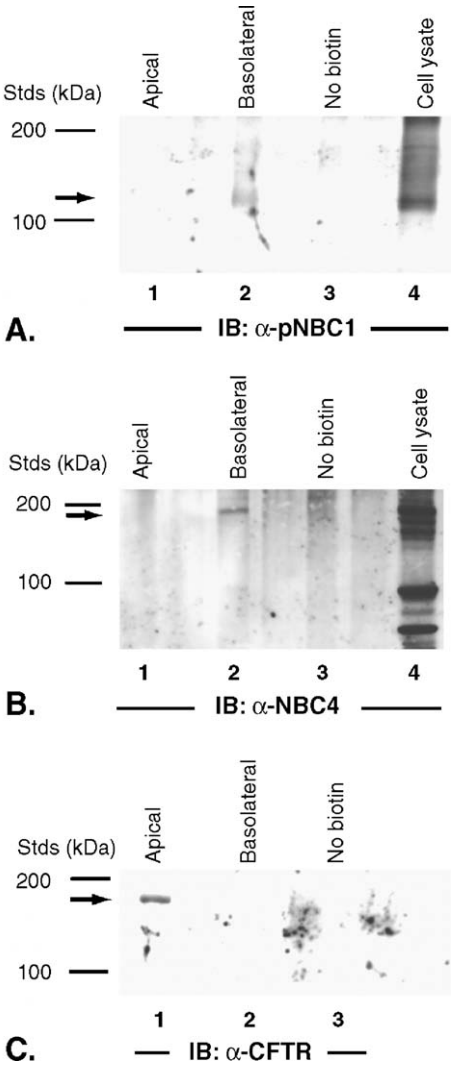

Fig. 5. Electrogenic NBCs in Calu-3 cells are localized to the basolateral membrane. Individual membranes were biotinylated and biotinylated proteins were captured using avidin-labeled agarose beads. Following isolation, captured proteins were separated by SDS-PAGE and immunoblotting was performed with α-pNBC1, α-NBC4, or α-CFTR antibody. (A) Immunoblotting with α-pNBC1 antibody: lane 1: apical membrane biotinylation; lane 2: basolateral membrane biotinylation; lane 3: no biotinylation; lane 4: whole cell lysate. (B) Immunoblotting with α-NBC4 antibody: lane 1: apical membrane biotinylation; lane 2: basolateral membrane biotinylation; lane 3: no biotinylation; lane 4: whole cell lysate. (C) Immunoblotting with α-CFTR antibody: lane 1: apical membrane biotinylation; lane 2: basolateral membrane biotinylation; lane 3: no biotinylation.

predicted molecular weight of approximately 120 kDa. The observed molecular weight is also higher than that seen in studies of NBC4c in rat that demonstrated a molecular weight of 145 kDa [26]. The high observed molecular weight may represent the combination of glycosylation and other post-translational modifications or the presence of an alternate splice variant that contains additional exons but shares the antigenic sequence used for antibody generation. In either case, previous studies also have demonstrated that the apparent molecular weight of NBC4 when detected by immunoblotting in human and rat skeletal muscle is approximately 190–200 kDa [27].

To investigate whether one or both proteins underwent post-translational modification, we treated immunoprecipitated protein with either endo H or PNGase. We found evidence that both pNBC1 and NBC4 had undergone complex post-translational glycosylation as evidence by resistance to endo H but sensitivity to PNGase. Interestingly, the molecular weight of pNBC1 appeared slightly higher (~145 kDa) in these experiments than in our immunoblotting experiments using the same antibody to probe cell lysates (~125 kDa). This discrepancy may be because the protein was isolated by immunoprecipitation versus simply being identified by immunoblotting from cell lysates, or it may be related to slight differences in gel preparation. There is also possibility that we immunoprecipitated a protein resulting from alternative splicing of NBC1 [24]. However, our full-length RT-PCR detected only a single band despite homology of our primers to the cDNA of reported splice variants. We confirmed that we were studying pNBC1 by demonstrating that the band of interest did not immunoprecipitate in the presence of competing antigenic peptide.

Having determined that both pNBC1 and NBC4 were expressed as mature proteins in Calu-3 cells, we proceeded to use surface biotinylation to identify their membrane localization. Both proteins could be detected by membrane-specific biotinylation in the basolateral membrane but not the apical membrane of Calu-3 cells. This result is consistent with the electrophysiological and pharmacological fingerprint of Calu-3 cells described by Devor and colleagues [14]. Therefore, we have both RNA and protein data that strongly support our hypothesis that electrogenic NBCs are present in the basolateral membrane of Calu-3 cells.

There are currently four NBC genes described in the literature. Notably, the nomenclature in the literature is quite confusing, and, therefore, we have shown GenBank accession numbers in Table 1 to clarify our usage.[2] It is interesting to discuss briefly the transport stoichiometry of NBC activity as it relates to the model proposed by Devor and colleagues [14]. The measured basolateral membrane potential of Calu-3 cells stimulated with forskolin to secrete HCO_3^- was −44 mV and hyperpolarized to −60 mV after stimulation with forskolin plus 1-EBIO [30]. 1-EBIO activates basolateral membrane potassium channels and thereby causes the basolateral membrane to hyperpolarize. Calu-3 cells when stimulated by forskolin and 1-EBIO secrete predominately Cl^- and not HCO_3^-. These results suggest that the NBC at the basolateral membrane has a reversal potential of less than −60 mV, which would correspond to a transport stoichiometry of 3 HCO_3^- to 1 Na^+. Conversely, a recent paper by Krouse and colleagues suggests that HCO_3^- secretion continues even after activation of Cl^- secretion by opening of basolateral K^+ channels [21].[3] In this case, one would predict that the NBC transport stoichiometry would be 2 HCO_3^- to 1 Na^+. Both pNBC1 and NBC4 have been studied in heterologous expression systems that lend insight into their transport stoichiometry. Studies on pNBC1 heterologously expressed in mouse renal proximal tubule cells suggest a stoichiometry of 3 HCO_3^- to 1 Na^+, whereas the same studies on pNBC1 performed in mouse cortical collecting duct cell line demonstrated a 2:1 stoichiometry, suggesting cell-type specific regulation [28]. Heterologous expression of NBC4c in *Xenopus laevis* oocytes has demonstrated both 3:1 [18] and 2:1 stoichiometry [23]. Therefore, it remains unresolved whether the stoichiometry of sodium bicarbonate cotransport in forskolin-stimulated Calu-3 cells is in agreement with that of the two candidate NBCs found by RT-PCR and western blotting, i.e., pNBC1 and NBC4c. Our studies do not directly address this question, and future studies will be required to elucidate the contribution of these transporters to HCO_3^- secretion in Calu-3 cells and human airway submucosal glands.

References

[1] H. Matsui, B.R. Grubb, R. Tarran, S.H. Randell, J.T. Gatzy, C.W. Davis, R.C. Boucher, Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease, Cell 95 (1998) 1005–1015.
[2] P. Linsdell, J.A. Tabcharani, J.M. Rommens, Y.X. Hou, X.B. Chang, L.C. Tsui, J.R. Riordan, J.W. Hanrahan, Permeability of wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride channels to polyatomic anions, J. Gen. Physiol. 110 (1997) 355–364.
[3] J.H. Poulsen, H. Fischer, B. Illek, T.E. Machen, Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 5340–5344.
[4] J.A. Tabcharani, J.M. Rommens, Y.X. Hou, X.B. Chang, L.C. Tsui, J.R. Riordan, J.W. Hanrahan, Multi-ion pore behaviour in the CFTR chloride channel, Nature 366 (1993) 79–82.
[5] H. Yue, S. Devidas, W.B. Guggino, The two halves of CFTR form a dual-pore ion channel, J. Biol. Chem. 275 (2000) 10030–10034.
[6] M.A. Gray, Bicarbonate secretion: it takes two to tango, Nat. Cell Biol. 6 (2004) 292–294.
[7] C. Clary-Meinesz, J. Mouroux, J. Cosson, P. Huitorel, B. Blaive, Influence of external pH on ciliary beat frequency in human bronchi and bronchioles, Eur. Respir. J. 11 (1998) 330–333.
[8] B. Holma, P.O. Hegg, pH- and protein-dependent buffer capacity and viscosity of respiratory mucus. Their interrelationships and influence on health, Sci. Total Environ. 84 (1989) 71–82.
[9] Y. Song, D. Salinas, D.W. Nielson, A.S. Verkman, Hyperacidity of

[2] For a review of the basic nomenclature used in this paper see article by Soleimani and Burnham [29]. Please note that further cloning and characterization of NBCs and of sodium-dependent anion exchangers has occurred since the publication of the review article.

[3] It is important to note that Krouse and co-workers performed their pH stat studies with a HCO_3^- free solution on the apical side where as Devor et. al. used HCO_3^- containing solutions on both sides of the epithelium. A HCO_3^- free solution on the apical side would allow for an artificial driving force for metabolic derived HCO_3^- exit across the apical and this may explain why Krouse et. al. observed both HCO_3^- and Cl^- secretion after stimulation of potassium channels.

secreted fluid from submucosal glands in early cystic fibrosis, Am. J. Physiol.: Cell Physiol. (2006) C741–C749.
[10] R.D. Coakley, B.R. Grubb, A.M. Paradiso, J.T. Gatzy, L.G. Johnson, S.M. Kreda, W.K. O'Neal, R.C. Boucher, Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 16083–16088.
[11] J.F. Engelhardt, M. Zepeda, J.A. Cohn, J.R. Yankaskas, J.M. Wilson, Expression of the cystic fibrosis gene in adult human lung, J. Clin. Invest. 93 (1994) 737–749.
[12] J. Jacquot, E. Puchelle, J. Hinnrasky, C. Fuchey, C. Bettinger, C. Spilmont, N. Bonnet, A. Dieterle, D. Dreyer, A. Pavirani, et al., Localization of the cystic fibrosis transmembrane conductance regulator in airway secretory glands, Eur. Respir. J. 6 (1993) 169–176.
[13] B.Q. Shen, W.E. Finkbeiner, J.J. Wine, R.J. Mrsny, J.H. Widdicombe, Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl-secretion, Am. J. Physiol. 266 (1994) L493–L501.
[14] D.C. Devor, A.K. Singh, L.C. Lambert, A. DeLuca, R.A. Frizzell, R.J. Bridges, Bicarbonate and chloride secretion in Calu-3 human airway epithelial cells, J. Gen. Physiol. 113 (1999) 743–760.
[15] C.J. Gottardi, L.A. Dunbar, M.J. Caplan, Biotinylation and assessment of membrane polarity: caveats and methodological concerns, Am. J. Physiol. 268 (1995) F285–F295.
[16] N. Abuladze, I. Lee, D. Newman, J. Hwang, K. Boorer, A. Pushkin, I. Kurtz, Molecular cloning, chromosomal localization, tissue distribution, and functional expression of the human pancreatic sodium bicarbonate cotransporter, J. Biol. Chem. 273 (1998) 17689–17695.
[17] A. Pushkin, N. Abuladze, I. Lee, D. Newman, J. Hwang, I. Kurtz, Cloning, tissue distribution, genomic organization, and functional characterization of NBC3, a new member of the sodium bicarbonate cotransporter family, J. Biol. Chem. 274 (1999) 16569–16575.
[18] P. Sassani, A. Pushkin, E. Gross, A. Gomer, N. Abuladze, R. Dukkipati, G. Carpenito, I. Kurtz, Functional characterization of NBC4: a new electrogenic sodium–bicarbonate cotransporter, Am. J. Physiol.: Cell Physiol. 282 (2002) C408–C416.
[19] S.K. Inglis, L. Finlay, S.J. Ramminger, K. Richard, M.R. Ward, S.M. Wilson, R.E. Olver, Regulation of intracellular pH in Calu-3 human airway cells, J. Physiol. 538 (2002) 527–539.
[20] A.W. Cuthbert, C.T. Supuran, L.J. MacVinish, Bicarbonate-dependent chloride secretion in Calu-3 epithelia in response to 7,8-benzoquinoline, J. Physiol. 551 (2003) 79–92.
[21] M.E. Krouse, J.F. Talbott, M.M. Lee, N.S. Joo, J.J. Wine, Acid and base secretion in the Calu-3 model of human serous cells, Am. J. Physiol.: Lung Cell. Mol. Physiol. 287 (2004) L1274–L1283.
[22] A. Pushkin, N. Abuladze, D. Newman, I. Lee, G. Xu, I. Kurtz, Cloning, characterization and chromosomal assignment of NBC4, a new member of the sodium bicarbonate cotransporter family, Biochim. Biophys. Acta 1493 (2000) 215–218.
[23] L.V. Virkki, D.A. Wilson, R.D. Vaughan-Jones, W.F. Boron, Functional characterization of human NBC4 as an electrogenic Na+-HCO3-cotransporter (NBCe2), Am. J. Physiol.: Cell Physiol. 282 (2002) C1278–C1289.
[24] M.O. Bevensee, B.M. Schmitt, I. Choi, M.F. Romero, W.F. Boron, An electrogenic Na(+)-HCO(−)(3) cotransporter (NBC) with a novel COOH-terminus, cloned from rat brain, Am. J. Physiol.: Cell Physiol. 278 (2000) C1200–C1211.
[25] A.B. Maunsbach, H. Vorum, T.H. Kwon, S. Nielsen, B. Simonsen, I. Choi, B.M. Schmitt, W.F. Boron, C. Aalkjaer, Immunoelectron microscopic localization of the electrogenic Na/HCO(3) cotransporter in rat and ambystoma kidney, J. Am. Soc. Nephrol. 11 (2000) 2179–2189.
[26] N. Abuladze, A. Pushkin, S. Tatishchev, D. Newman, P. Sassani, I. Kurtz, Expression and localization of rat NBC4c in liver and renal uroepithelium, Am. J. Physiol.: Cell Physiol. 287 (2004) C781–C789.
[27] J.M. Kristensen, M. Kristensen, C. Juel, Expression of Na+/HCO3-co-transporter proteins (NBCs) in rat and human skeletal muscle, Acta Physiol. Scand. 182 (2004) 69–76.
[28] E. Gross, N. Abuladze, A. Pushkin, I. Kurtz, C.U. Cotton, The stoichiometry of the electrogenic sodium bicarbonate cotransporter pNBC1 in mouse pancreatic duct cells is 2 HCO(3)(−):1 Na(+), J. Physiol. 531 (2001) 375–382.
[29] M. Soleimani, C.E. Burnham, Na+:HCO(3−) cotransporters (NBC): cloning and characterization, J. Membr. Biol. 183 (2001) 71–84.
[30] T. Tamada, M.J. Hug, R.A. Frizzell, R.J. Bridges, Microelectrode and impedance analysis of anion secretion in Calu-3 cells, J. Pancreas 2 (2001) 219–228.